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Development of vaccines and their use in the prevention of fungal infections

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Vaccine approaches to infectious diseases are widely applied and appreciated. Disciplines such as bacteriology and virology have a rich history of successful vaccine development. The complexity of eukaryotic systems presents additional challenges to the development of vaccines against them. These challenges are being met in the fields of parasitology, and are being revisited for application in oncology. Vaccine opportunities exist in medical mycology. The National Institute of Allergy and Infectious Diseases has held a series of workshops in medical mycology where the need to develop vaccines for fungal diseases was noted and where important opportunities were discussed. Major advances in vaccinology and the technology of antigen preparation and delivery have increased feasibility and heightened interest. The recent epidemic of coccidioidomycosis in the American Southwest has demonstrated the need for developing a vaccine as an effective preventive measure for those living in and for those who subsequently move into regions with the endemic mycoses. The XIIth Congress of the International Society for Human and Animal Mycology included a symposium that summarized new vaccination strategies for selected fungi: *Candida albicans*, *Coccidioides immitis*, and *Trichophyton verrucosum*. The goal of the present summary is to provide representative examples of continuing efforts relating to vaccine development within the medical mycological community highlighting *Blastomyces dermatidis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Pythium insidiosum*.

Keywords vaccines, prevention, mycoses, pythiosis, immunization, therapeutic

Introduction

The use of vaccines in preventing infection has been applied to a broad range of infectious diseases. Disciplines such as bacteriology and virology have a rich history of successful vaccine development [1]. The complexity of eukaryotic systems, however, presents additional challenges to the development of vaccines in this field. These challenges are already being met in parasitology and are receiving much attention in oncology.

Many opportunities exist for vaccine development in medical mycology. The National Institute of Allergy and Infectious Diseases has held a series of workshops in medical mycology where the need to develop vaccines for the prevention of fungal diseases was noted and important opportunities for this were discussed [2,3]. Major advances in vaccinology and the technology of antigen preparation and delivery have increased the feasibility and heightened interest in this approach in medical mycology [4]. The recent epidemic of coccidioidomycosis in the American south-west has demonstrated the need for a vaccine as an effective preventive measure for those living in, or moving to, regions with endemic mycoses [5].

The XIIth Congress of the International Society for Human and Animal Mycology included a symposium that summarized new vaccination strategies for *Candida albicans*, *Coccidioides immitis* and *Trichophyton verrucosum*.

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The goal of the present summary is to provide representative examples of fungal infections where efforts to develop vaccines are continuing. These include infections caused by the fungal pathogens *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Pythium insidiosum*.

Histoplasmosis

In previous reports, two groups of investigators demonstrated that immunization with extracts from *H. capsulatum* could protect mice from subsequent infection. An ethylenediamine extract from the cell wall and cell membrane of this fungus was protective to mice [6]. In addition, a ribosomal-protein complex also induced protective immunity; the activity of this complex was dependent on the protein portion [7]. Unfortunately, no studies have succeeded in isolating a single molecular species that could exert immunological activity that was identical to the extracts.

With this background, Deepe and coworkers postulated that a protein(s) from this fungus could induce a protective immune response. A series of studies was initiated in an attempt to identify an antigen(s) that could be potentially useful as a vaccine. A detergent extract was prepared from the cell wall and cell membrane of *H. capsulatum*, that was demonstrated to stimulate cellular immune responses in mice and humans. This extract induced proliferation of T-cells from mice immunized with either *H. capsulatum* yeast cells or with the extract [8].

Immunization experiments were conducted to determine if the crude extract was protective against experimental infection in animals. Immunization with the extract emulsified in adjuvant protected C57BL/6 mice from a lethal intravenous injection of *H. capsulatum* [8]. Subsequently, the specific antigens responsible for this protective effect were sought from the crude extract. T-cell Western blotting was used to map the immunogenic determinants in the cell wall and cell membrane extracts [8]. Two T-cell lines and five of six T-cell clones from C57BL/6 mice responded to a region that spanned 59–67 kDa in size [8]. A protein, termed HIS-62, was purified from this region [9] and the immunological properties of this protein were analysed. A high proportion of monoclonal T-cells from C57BL/6, BALB/c and CBA/J mice recognized this protein. Moreover, it could induce cutaneous delayed-type hypersensitivity (DTH) responses in mice exposed to *H. capsulatum* yeast cells. Since this protein was associated with a cellular immune response, it was of interest to determine whether it could protect mice from a lethal intravenous injection of yeast cells. A high proportion (> 80%) of BALB/c, CBA/J and C57BL/6 mice immunized with HIS-62 in the presence of adjuvant survived for over 30 days whereas none of the controls lived beyond day 15 [9]. Thus, a protein had been isolated from *H. capsulatum* that could induce protective immunity.

Deepe's group also investigated whether HIS-62 was recognized by sera from humans who had been exposed to *H. capsulatum*. Peripheral blood mononuclear cells from seven *H. capsulatum*-exposed individuals recognized the cell wall and cell membrane extracts as well as HIS-62. A high proportion (≈ 85%) of human T-cell clones maintained with the extract reacted to HIS-62. Thus, this antigen was stimulatory for human as well as murine cells [10].

The focus then turned to the identity of this protein. HIS-62 was cleaved with cyanogen bromide and the fragments were transferred to polyvinylidene difluoride membranes and subjected to amino acid sequencing. Degenerate oligonucleotides were synthesized based on the amino acid sequence and used in the polymerase chain reaction to amplify a fragment of the gene encoding HIS-62. A gene segment was isolated and sequenced, and the DNA sequence of this fragment suggested homology to the heat shock protein (hsp) 60 family. The full-length gene was isolated from a genomic library of *H. capsulatum* sequenced in both directions. The deduced amino acid sequence of the gene was 50–70% identical to prokaryotic and eukaryotic members of the hsp 60 family. Thus, the data indicated that HIS-62 was the *H. capsulatum* homologue of hsp 60 [11].

A recombinant protein was generated and tested for its immunogenicity. The recombinant protein stimulated lymphocytes from BALB/c mice immunized with yeast cells and from mice immunized with the protein itself. Moreover, injection of recombinant hsp 60 conferred a protective immune response in mice challenged intranasally with a lethal inoculum of yeast cells [11]. Thus, recombinant hsp 60 mimicked the activity of the protein isolated from *H. capsulatum*.

It was surprising that this highly conserved antigen was protective. One would have expected that a protein that had little homology to mammalian proteins would be a more likely candidate as a protective immunogen. Nevertheless, hsp 60 from *Legionella pneumophila* has been shown to protect guinea-pigs from a lethal aerosol challenge [12]. Furthermore, genetic vaccination with the *Mycobacterium tuberculosis* gene encoding hsp 65 can protect mice against this bacterium [13]. Thus, there is now a growing body of evidence that highly conserved proteins can be protective in various infections.

Since hsp 60 was demonstrated to be protective, attempts were made to determine if the protective efficacy of this protein was segregated into a particular domain. Four overlapping fragments of the gene were generated and expressed in the eukaryotic expression vector,

pET19b. Recombinant polypeptides were purified and tested for their ability to induce proliferative responses in BALB/c and C57BL/6 mice immunized with either *H. capsulatum* yeast cells or with recombinant *H. capsulatum* hsp 60. Each fragment induced proliferation of splenocytes from mice immunized with recombinant hsp 60 although differences between the two strains were observed in the magnitude of the stimulatory activity. Unexpected findings were discovered in mice immunized with yeast cells. Splenocytes from BALB/c mice responded to the fragments whereas cells from C57BL/6 mice did not, although native hsp 60 did induce proliferation. This finding remains unexplained [14].

Both strains of mice were immunized with each fragment and challenged intranasally with a sublethal inoculum of yeast cells ($2{\cdot}5 \times 10^6$ yeasts); lungs, spleens and livers were cultured 1 week after infection. This time point was selected because it is the peak time of infection with this inoculum size. Of the four fragments, fragment 3 induced protective immunity in both strains of mice. Mice were subsequently immunized with each of the fragments and tested for their ability to survive a lethal intranasal challenge. Fifty percentage of either strain of mouse immunized with fragment 3 survived for over 30 days, whereas the remainder died within 20 days [14]. Thus, the protective domain of hsp 60 was located to this polypeptide.

It was somewhat disappointing that only 50% of the mice immunized with fragment 3 survived. The lack of efficacy of this fragment compared to the whole protein may be explained by the fact that the inoculum size was doubled in this mouse challenge study in order to highlight the efficacy of the fragments. Alternatively, hsp 60 from *M. tuberculosis* has been shown to contain adjuvant-like activity [15] and it may well be that optimal efficacy of hsp 60 from *H. capsulatum* requires a stretch of the protein that also contains adjuvant activity.

In parallel with the above studies, two additional recombinant proteins were produced from *H. capsulatum*, H antigen and hsp 70. Although both of these recombinant proteins elicited a cellular immune response in mice, neither of them exerted a protective effect [16,17]. Thus, although a protein can induce a cellular immune response in mice, it does not necessarily possess the properties to protect mice from subsequent challenge. It is possible that either of these two genes may be protective if they are used as genetic vaccines as is the case with *M. tuberculosis* hsp 60 [13]. The mounting data suggest that not all proteins that evoke a cellular immune response are protective. The critical issue is to identify what properties of a protein make it protective. One likely explanation is that the cytokine profile produced in response to a protein may determine the protective efficacy of an immunogen. On the other hand, the quantity of cytokines generated in response to a vaccine may influence its protective properties. These two postulates are not mutually exclusive. Further studies are in progress to address these hypotheses using both non-protective and protective proteins.

Paracoccidioidomycosis

Human paracoccidioidomycosis (PCM) is a chronic systemic disease characterized by suppurative granulomatous inflammation, suppression of cellular immunity and high antibody titres [18]. The dimorphic fungus, *P. brasiliensis*, is the aetiological agent of this mycosis. A high incidence of PCM is found in Latin America, particularly in tropical and subtropical areas of Brazil, where young rural workers are most often affected.

Airborne fungal propagules are thought to initiate the infection in the lung after conversion to the yeast phase. The infection can proceed either as a mild, self-limited process, or be severe and progressive, eventually fatal, spreading to extra-pulmonary tissues. In the benign form, cellular immunity is preserved with positive intradermal reactions to paracoccidioidin, formation of compact epithelioid granulomas and moderate specific antibody responses. In disseminated infection, the anergic clinical form is characterized by a decreased or negative cellular immune response and loose granuloma formation with a great number of yeast cells [19–21]. Depression of cell-mediated immunity in PCM is a reversible process [22] and clinical remission of the mycosis is often followed by reacquisition of positive skin tests. The high titre antibody response is not protective, although it may include specific antibodies that neutralize virulence factors such as proteinases and matrix-binding fungal components.

Few attempts at immunization against PCM have been carried out. Protection has always been correlated with the presence of a vigorous cellular immune response, limited infection and fewer fungal cells in the granulomata [23]. The protective role of the major diagnostic antigen of *P. brasiliensis*, the 43 kDa glycoprotein (gp43) [24,25], is currently being investigated. Virtually all patients with PCM have antibodies against gp43 and the epitopes recognized are mostly peptide in nature [25,26]. gp43 is secreted continuously by growing yeast cells [27] and is retained *in vivo* within macrophages in the area of inflammation [28]. Although gp43 is highly immunogenic for antibody production, it is also immunodominant in eliciting DTH responses [29]. Sensitized mice respond to gp43 by proliferation of CD4 +, but not CD8 +, cells [30]. gp43-reactive CD4 + cells are of the T-helper-1 (Th-1) type which produce interferon (IFN)-γ but not interleukin (IL)-4 or-10 (C. Taborda & L. R. Travassos, unpublished results). The secretion of IFN-γ was much enhanced by recombinant IL-2. The gp43 gene has been cloned [31] and

sequenced completely. It showed 56–58% similarity to the *C. albicans* and *Saccharomyces cerevisiae* genes encoding exo-β-1,3-D-glucanases, although a point mutation in the the tripeptide sequence asparagine, glutamic acid, proline inhibits its hydrolytic activity. This antigen may play a role in fungal virulence. gp43 binds specifically to laminin-1 and the fungal yeast cells coated with laminin are several times more invasive and destructive than untreated cells in a hamster-testicle infection model [32]. Monoclonal antibodies were shown to modulate gp43-mediated fungal invasion in the presence of laminin [33]. Susceptible B10.A mice infected with 5×10^5 virulent yeast cells by intratracheal inoculation contained increased numbers of colony forming units (CFUs) in their lungs and livers only when the yeast suspension inoculated also contained 50 g of gp43 (C. Taborda & L. R. Travassos, unpublished results). It appears then that gp43 is a virulence factor and that an immune response against it could be protective.

Since both gp43 and a 15 amino acid peptide derived from it (P10) are strong elicitors of IFN-γ production by Th-1 CD4 + cells, one may ask how important is IFN-γ in the evolution of experimental PCM. Pulmonary macrophages activated by IFN-γ *in vivo* and *in vitro* enhanced killing of *P. brasiliensis* [34]. Mice homozygous for the 'null' mutation of the gene encoding the IFN-γ receptor [35] were inoculated intratracheally with 5×10^5 yeast cells of the Pb18 strain. After 5 weeks of infection all mutants died with progressive disease, in contrast to the wild-type animals that were resistant to the infection in the same period. Lung granulomas in the wild-type were typically epithelioid with few neutrophils. In contrast, granulomas in the mutants were looser, had more exudate and were rich in neutrophils. The infection was restricted to the lungs in the wild-type, whereas in the knock-out mice dissemination was observed in the liver and spleen with suppurative, exudative lesions containing several yeast cells in anergic-type granulomas (C. Taborda & L. R. Travassos, unpublished results). Thus, IFN-γ is able to determine the course and severity of infection by *P. brasiliensis*. Since gp43 elicits IFN-γ production in sensitized animals and seems to be a virulence factor, the question is raised regarding the role of gp43 and that of its immunodominant epitope P10, in protection against experimental PCM. In a short-term experiment (1 month), intratracheally inoculated (3×10^5 virulent yeast cells), BALB/c mice, immunized previously with gp43 or P10, exhibited significantly reduced CFUs in the lungs. After 3·5 months of infection, the number of CFUs in immunized animals decreased more than 200 times in the lungs and, in contrast with the control animals, immunization with gp43 or P10 also prevented fungal dissemination to the spleen and liver (C. Taborda, L. Juliano & L. R. Travassos, unpublished results).

While the above results indicate a protective role for gp43 in a murine infection system, it is still unknown whether this molecule, which carries B-cell epitopes and induces IFN-γ production in IL-2-stimulated CD4 + lymphocytes, or some of its peptide epitopes (e.g. P10) can revert the anergic state in chronic PCM which is related to the fungal load and may involve other antigens and immune responses.

Blastomycosis

Blastomycosis, one of the principal endemic systemic mycoses, is caused by the thermal dimorphic fungus, *B. dermatitidis*. Humans and other mammals are infected primarily by inhaling aerosolized conidia from the mould in soil, where the organism is believed to dwell as a saprophyte [36]. At a temperature of 37 °C in the host, conidia transform into the pathogenic yeast phase, which multiplies within the lung, and may disseminate via the bloodstream and lymphatic system to visceral organs [37]. The acute primary pulmonary infection with *B. dermatitidis* may be asymptomatic or produce an influenza-like illness or atypical pneumonia. Acute infection may resolve spontaneously, but progressive disease of the lung, extra-pulmonary organs, or both, develops in many patients.

Epidemiological features are not understood completely [38]. Blastomycosis occurs mainly as a sporadic infection in immunocompetent hosts, but many epidemics and cases of opportunistic infection have been described in patients with acquired immunodeficiency syndrome and other immunocompromised hosts [39]. A distinguishing feature of blastomycosis is the high frequency of clinical disease compared with mild and asymptomatic infections, highlighting the pathogenicity of the organism. Blastomycosis is a common infection in dogs who reside in endemic zones [40]. The severity and lethality of canine infection also highlights the pathogenicity of *B. dermatitidis*.

B. dermatitidis evokes a vigorous, innate inflammatory response characterized by an influx of neutrophils and mononuclear phagocytes. At these inflammatory foci, *B. dermatitidis* yeasts are present both inside and outside the phagocytes. *In vitro* studies have shown that yeasts replicate readily in macrophages until they are activated by cytokines from T-cells [41]. Within several weeks after natural infection of humans and experimental infection of animals, the host develops acquired immunity to *B. dermatitidis*. This is evidenced by the appearance of DTH [42], proliferation of T-cells *in vitro* [43], and circulating antibodies in response to antigens of the fungus [36]. In a murine model of blastomycosis, passively transferred T-cells, but not serum, from immune to naive animals conferred protection, suggesting that immunity resides chiefly with antigen specific T-cells [44,45]. No vaccine studies

have been reported with *B. dermatitidis*. However, a vaccine would be valuable for preventing canine and possibly human blastomycosis. Vaccine studies in dogs could evaluate new strategies that might also be useful in developing fungal vaccines for use in humans. These studies will demand a clear understanding of the antigens of *B. dermatitidis* and of the correlates of protective immunity to the fungus.

Work in recent years has elucidated the identity of target antigens of humoral and cellular immune responses to the fungus [46]. Until recently, the major antigens characterized for *B. dermatitidis* included A-antigen and *Blastomyces* alkali-and water-soluble antigen (B-ASWS). A-antigen is a 98 kDa glycoprotein derived from yeast culture autolysates [47]. It has been used extensively for detecting antibodies during human and animal infection. Most commercial test kits, however, have a high frequency of cross-reactivity due to carbohydrates on the molecule, which contain epitopes recognized by antibodies in patients with other fungal infections. B-ASWS, a crude preparation derived by alkali extraction of yeast cell walls, contains determinants that evoke DTH responses in *Blastomyces*-sensitized guinea-pigs [48] and stimulate *in vitro* proliferation of lymphocytes taken from infected humans [49]. Klein and coworkers have shown that a 120 kDa protein on the surface of *B. dermatitidis* yeasts, designated WI-1, contains the antigenic determinants that antibodies recognize on A-antigen and that T-cells recognize on B-ASWS [49,50].

WI-1 is highly conserved in the yeast form of *B. dermatitidis* [51] and is secreted by the yeast in large amounts during culture *in vitro* [52]. Cloning and functional characterization of the protein have shown that WI-1 is an adhesin that attaches the fungus to human macrophages by binding to CR3 and CD14 receptors [53,54]. Surface expression and secretion of WI-1 are altered significantly on genetically related strains of the fungus that differ in their virulence to mice [52], raising the possibility that the adhesin serves as a virulence factor in *B. dermatitidis*.

Most humans infected with the fungus produce strong antibody and T-cell responses to WI-1. Studies in Wisconsin have shown that almost 90% of patients recognize and respond to the protein with both limbs of the immune system [49,51]. Dogs also mount an immune response to WI-1, as evidenced by the presence of circulating antibodies during infection in over 90% of infected animals. Work in a murine model of blastomycosis has confirmed the immunodominance of WI-1; infected mice demonstrate specific antibodies and DTH or *in vitro* lymphocyte responses to the antigen (see below).

Molecular cloning and sequencing has shown that WI-1 contains three domains [46,53]: (i) a central region consisting of a 25 amino acid repeat, arrayed in tandem and present in 34 copies. The tandem repeat has homology with *Yersinia* invasin, an adhesin and virulence determinant, and also binds the fungus to receptors on macrophages, (ii) an epidermal growth factor-like domain at the C-terminus, a sequence motif that governs adhesive interactions with extracellular matrix components, and (iii) a hydrophobic sequence at the N-terminus that may insert WI-1 in the fungal cell membrane.

The WI-1 epitopes recognized by antibodies and T-cells during human infection have been identified [50,55,56]. In addition, the major histocompatibility complex antigens that bind and display these epitopes to human CD4 + T-cells have been characterized. Humans who have been infected with *B. dermatitidis*, and rabbits and mice who have been immunized with WI-1, produce antibodies targeted almost entirely against the 25 amino acid repeat [56]. In an antigen-inhibition radioimmunoassay, the binding of polyclonal antisera from either infected humans or immunized rabbits to radiolabelled WI-1 was inhibited by 90% by blocking with the 25 amino acid repeat [56]. Of 12 BALB/c mouse-derived monoclonal antibodies reactive with WI-1, 10 were directed against the 25 amino acid repeat [50]. The reason for this focused immune response is not known. Antibodies may interfere with attachment of the fungus to host cells and this could represent an immunological defence mechanism. It is interesting that the 25 amino acid repeat is also displayed on the A-antigen and represents the specific target of antibodies directed against that antigen [50].

CD4 + T-cells cloned from the peripheral blood of infected humans responded to WI-1 determinants shared with the B-ASWS antigen of *B. dermatitidis* [49]. These cloned T-cells can therefore be used to identify epitopes that evoke DTH reactions to the fungus, responses which are presumably essential in protective immunity. Klein and coworkers have mapped WI-1 T-cell epitopes and found that they were displayed by HLA-DR and HLA-DP antigens on antigen presenting cells. Epitopes recognized by HLA-DR restricted T-cells were located in the amino terminus of WI-1, between residues 116–143 [55], whereas epitopes recognized by HLA-DP restricted T-cells were found in the 25 amino acid tandem repeat. In functional assays, HLA-DR restricted T-cells were non-cytolytic, whereas HLA-DP restricted T-cells lysed targets that displayed WI-1 on their surface [49]. These findings suggest that major histocompatibility complex selection of epitopes may influence the profile and function of cell-mediated immunity to *B. dermatitidis*.

The foregoing studies have addressed the antigenicity of WI-1 and indicate that humans and other species recognize and respond to WI-1 during the course of natural infection. Based on these observations, it should be possible to induce an immune response by immunizing experi-

mental animals with WI-1; it should then be possible to investigate the protective efficacy of anti-WI-1 immunity in blastomycosis. For these studies, a murine model of blastomycosis was adapted as described previously [57]. Six-week-old, male, C57BL/6 mice were immunized with WI-1 and then challenged intranasally with a lethal dose of yeasts to assess the protective efficacy of WI-1 immunization. To ascertain the WI-1 dose yielding the most robust immune response, varying amounts of the antigen ranging from 5 to 200 mg were administered subcutaneously in complete Freund's adjuvant; this was then repeated 2 weeks later in incomplete Freund's adjuvant. Two weeks after the last immunization, animals were tested for their immune response as determined by: (i) the magnitude of the DTH response to WI-1 injected into the footpads, (ii) antibody titres to WI-1, and (iii) the profile of anti-WI-1 IgG subclasses, which indirectly 1vs. Th2 responses to the antigen. A concentration-dependent effect of the immunizing dose of the antigen was observed. The largest doses produced the strongest DTH responses and highest antibody titres. At all immunizing doses, the anti-WI-1 IgG response was comprised mainly of IgGl and IgG2b subclasses, followed by IgG3 and IgG2a. These IgG subclasses illustrate that a Th2 response predominated over a Th1 response. The same composition of anti-WI-1 IgG subclasses was observed during an experimental murine infection.

Immunization with WI-1, as above, has been shown to protect mice from a lethal challenge with *B. dermatitidis*. Mice were immunized with 100 μg of WI-1 using the schedule outlined above. Immunized mice were then challenged intranasally with a lethal dose of 10^4 yeasts (ATCC strain 60636) and their survival was measured over the ensuing 30 days. Six (75%) of eight mice immunized with WI-1 survived the infection, whereas none of the eight control mice immunized with bovine serum albumin survived ($P = 0{\cdot}0002$, log rank test). Histopathology of lung tissue from WI-1-immunized and control mice showed heavy infection and inflammation in the control mice, but only mild inflammation and sparse organisms in the WI-1-immunized mice. These findings indicate that anti-WI-1 immunity protects against experimental lethal infection and that the antigen is a promising candidate as a vaccine against blastomycosis.

A future goal is to investigate the correlates of WI-1-mediated protective immunity. It is proposed that anti-WI-1 antibodies and T-cells cooperate in mediating protection. Although cellular immunity is believed to be paramount in protecting the host against this fungus, the humoral immune response to *B. dermatitidis*, and, in particular, antibodies against the 25 amino acid repeat, may offer the host a selective advantage against infection by interfering with attachment or by promoting uptake of the fungus into activated phagocytes. Defining the correlates of protective immunity will provide new information about the immunobiology of blastomycosis and will yield cellular reagents for elucidating the structural basis of protective immunity (i.e. B-and T-cell epitopes that drive protective immune responses). This information will allow us to develop vaccines that harness both limbs of the immune response by incorporating B-and T-cell epitopes into chimeras fused in the form of a synthetic peptide or DNA. Such vaccines may protect dogs against blastomycosis and could illuminate new strategies for preventing this infection in humans.

Pythiosis

Pythiosis is a cutaneous, subcutaneous and systemic disease of humans and lower animals caused by the fungus-like organism *P. insidiosum* [58]. The disease was described at the beginning of the century in horses in tropical and subtropical countries including India and Indonesia, as well as the USA. Soon, however, it was evident that the disease not only affected horses but also other mammalian species [59]. *P. insidiosum* is an organism belonging to the Kingdom Chromista, Phylum Pseudofungi, in the Class Oomycetes. *P. insidiosum* is not only phylogenetically distinct from the members of the Kingdom Fungi but it also differs physiologically. This may explain why the drugs use to treat the mycoses caused by the opportunistic and pathogenic fungi do not have any effect on pythiosis. Consequently, new ways to treat this disease were investigated several years ago [60,61].

The finding that immunogens of *P. insidiosum* possessed curative properties was first noticed when horses with pythiosis from Costa Rica were cured following injection with *P. insidiosum* antigens that were used for skin testing [60]. Simultaneously, a similar vaccine with curative properties was used successfully in horses with the disease in Australia [61]. These two vaccines have been referred to in the literature as Mendoza's and Miller's vaccines, respectively [62]. Early reports indicated that the antigens used in the *P. insidiosum* vaccine possessed unique characteristics, somewhat similar to the features of those reported in *T. verrucosum* [63] and other immunotherapeutic vaccines [64,65].

Miller's vaccine consists of sonicated hyphal antigens [61], while Mendoza's vaccine is prepared from culture filtrate antigens [60]. Both vaccines have cured ≈ 53% of vaccinated horses. Mendoza's vaccine, however, has a longer shelf-life and milder side-effects [66]. In addition to its immunotherapeutic features, Mendoza's vaccine also showed some degree of protection which was later found to be of short duration [67]. In 15 years of use, more than 300 cases of pythiosis in horses have been cured with this

vaccine which has been shown to be both consistent and safe. However, the vaccine only cured early equine pythiosis and not chronic cases of this disease [44].

Increasing reports of pythiosis in humans, and the success in treating equine pythiosis with Mendoza's vaccine, have motivated experiments to investigate the proteins involved in the immunoprotective and immunotherapeutic effects of this vaccine. In a recent study using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, three immunodominant hyphal proteins were found [68]. Immunoblotting revealed that the IgG in sera from horses with active pythiosis recognized most of the proteins in *P. insidiosum*. However, of all the proteins analysed, three bands of 32 kDa, 30 kDa and 28KDa, were particularly prominent. More significant was the finding that antibodies against these three proteins persisted for long periods of time in the horses that had been vaccinated successfully [68]. These data suggested that the three prominent proteins possessed protective and therapeutic features and that they would be good candidates for vaccination trials.

Additional SDS-PAGE analyses were conducted on the three immunodominant *P. insidiosum* hyphal proteins. Following electrophoresis, the prominent proteins were cut from the acrylamide gels and purified. A mixture of the three proteins was added to Mendoza's original vaccine ($\approx 2{\cdot}0$ mg ml^{-1} final concentration). A Western blot analysis was then performed on the vaccine to verify the presence of the three proteins. The vaccine was then stored at 4 °C until use.

One major drawback in evaluating the *P. insidiosum* vaccine is the lack of a laboratory animal model of this infection. The only animal in which the disease can be reproduced successfully is the rabbit (*Orcytologous cuniculus*). However, no systematic studies have been conducted to evaluate its effectiveness as an experimental model. Evaluation of the *P. insidiosum* vaccine has been carried out only in horses with the disease. Neither Miller's nor Mendoza's vaccines can cure infected horses after 60 days or more of infection. Therefore, studies with the purified hyphal proteins were conducted in both acute and chronic stages of equine infection. Seven horses were selected with chronic pythiosis (disease > 60 days duration, some > 100 days duration) and three with acute pythiosis (disease < 60 days duration). The diagnosis of pythiosis in the treated horses was verified either by serology and/or culture, and by histopathology. All animals were vaccinated with Mendoza's vaccine supplemented with the three purified hyphal proteins. Results indicated that the addition of these three immunodominant proteins enhanced the curative properties of the Mendoza vaccine remarkably. This vaccine cured all of the early cases of pythiosis. Of the seven vaccinated horses with chronic pythiosis, four were cured, two did not respond and one responded initially but subsequently died. All of the cured horses developed a mild inflammatory reaction at the vaccination site. However, the three horses that did not respond to the vaccination did not develop such a reaction. Those horses with chronic infections exceeding 100 days duration were considered to be anergic.

The results of this study suggested that: (i) the addition of the three immunodominant proteins directly enhanced the curative properties of Mendoza's original vaccine which always failed in chronic cases (> 60 days) [67], (ii) these proteins are involved directly with the immunotherapeutic properties of the Mendoza vaccine, and (iii) these proteins play a role in the immunology of *P. insidiosum* infection.

These findings also confirmed previous speculation that the response to *P. insidiosum* vaccination is related directly to the immune status of the infected horse. Although the main attribute of the modified vaccine is its ability to cure chronic equine pythiosis, it retained all of the properties of the original vaccine. These include the production of a mild inflammatory reaction at the site of vaccination in cured but not in unresponsive horses, and 100% cure in early cases. The rate of cure with Mendoza's original vaccine was 53%, whereas after addition of the 32 kDa, 30 kDa and 28 kDa proteins, the cure rate increased to 70%. This enhancement of the curative properties of the vaccine was related directly to the addition of the three prominent proteins to the original vaccine.

Based on these promising results, Mendoza's group is now planing to clone the genes that encode these three major proteins to dissect, at a molecular level, the components behind its protective and curative properties. The data accumulated after several years of research indicate that this immunotherapeutic vaccine is effective, not by boosting the immune system of the infected host, as has been suggested [69], but, perhaps, by presentation of epitopes previously shielded in the masses formed after degranulation of eosinophils by *P. insidiosum* hyphae. The report that some horses were cured when degranulation of eosinophils was suppressed by the use of intramuscular steroids tends to support this hypothesis [70]. Should this new concept be correct, the development of similar immunotherapeutic vaccines against other eukaryotic microorganisms, using their unexposed immunogens as antigens, may be feasible. This enhanced vaccine has recently been used to successfully treat a Thai boy with systemic pythiosis arteritis. Details of this case are being prepared for publication.

Cryptococcosis

C. neoformans is unusual among fungal pathogens in that it has a polysaccharide capsule. The capsule is antiphagocytic and may function in virulence in a manner similar to

that of the classical encapsulated bacterial pathogens *Haemophilus influenzae* and *Streptococcus pneumoniae* [71]. Phagocytosis is an important host defence mechanism because internalization in phagocytic cells results in enhanced killing of fungal cells. In the case of *C. neoformans*, immune effector cells are unable to phagocytose encapsulated yeasts in the absence of opsonins. Unfortunately *C. neoformans* infection may be accompanied by a lack of opsonins since the polysaccharide capsule is poorly antigenic and often fails to elicit an antibody response [72], and acquired complement deficiency may result in a depletion of complement components [73].

Cell-mediated immunity is generally acknowledged to provide the main defence against *C. neoformans* infection. However, conditions which predispose to cryptococcosis such as late human immunodeficiency virus (HIV) infection, haematological malignancies and corticosteroid use are each characterized by profound derangements of both cellular and humoral immunity. The precise immune deficits that predispose to cryptococcal infection are unknown although it is likely that susceptibility to infection requires multiple immune deficits. The association between cryptococcosis and late CD4 + T-cell lymphopaenia that accompanies late HIV infection, could also reflect the central role of CD4 + cells in recruiting and activating phagocytic cells and/or coordinating effective antibody responses.

The role of natural antibody immunity in protection against *C. neoformans* is uncertain [74]. The anticryptococcal conjugate vaccine is based on the premise that a protective antibody response can be elicited even if antibody immunity plays only a minor role in protection against natural infection. This premise is supported by experience with antitoxin vaccines which are often protective by eliciting immune responses unlike those which accompany natural infection. For example, immunization with tetanus toxoid (TT) is protective against tetanus even though a bout of tetanus may not produce lasting immunity [75]. Hence, recovery from clinical tetanus many not elicit protective immunity but TT immunization is protective by eliciting neutralizing antibodies which prevent the clinical manifestations of *Clostridium tetani* infection. For *C. neoformans*, the aim would be to elicit protective antibody responses which prevent cryptococcosis. The conjugate vaccine against *C. neoformans* represented a major departure from conventional thinking on antifungal vaccines since, for the first time, a vaccine which elicited only antibody immunity against a fungal pathogen was designed, synthesized and tested.

The covalent linkage of cryptococcal glucuronoxylomannan (GXM) to TT results in a powerful immunogen (GXM-TT) which elicits high titre antibody responses in mice [76–78] and humans [79]. The molecular genetics of the antibody response to GXM-TT has been studied extensively in mice [80]. GXM-TT immunization of BALB/c mice elicited a restricted antibody response characterized by the expression of a combination of heavy and light chain variable genes, and an idiotype defined by the 2H1 monoclonal antibody [80,81]. In contrast to the polysaccharide antigen alone, the GXM-TT vaccine behaved as a T-cell dependent antigen and elicited predominantly IgG isotype antibodies [76]. Analysis of human monoclonal antibodies generated from volunteers immunized with GXM-TT suggested that the antibody response in humans is also restricted to variable gene utilization and idiotype [82,83]. The occurrence of variable gene restriction implies relative homogeneity in the molecular antibody response, a phenomenon that can have significant consequences depending on the protective efficacy of the antibodies made.

Considerable insight into the functional efficacy of the conjugate vaccine antibody response has been obtained in studies of murine and human monoclonal antibodies generated in response to GXM-TT vaccination. In mice, the protective efficacy of monoclonal antibodies to the capsular GXM depends on the antibody isotype [84] and epitope specificity [85], and the GXM-TT vaccine can elicit both protective and non-protective monoclonal antibodies which vary in epitope specificity [85]. This suggests that the GXM-TT vaccine contains epitopes capable of eliciting protective and non-protective antibodies. The ability of GXM-TT to elicit functionally useful and useless antibodies in mice may have a parallel in humans; immune human sera can exhibit significant differences in opsonin power which do not correlate directly with antibody titre [86].

Murine and human monoclonal antibodies elicited by GXM-TT vaccination can be potent opsonins [86,87]. In murine experimental pulmonary infection, antibody administration has been associated with prolongation of survival and enhanced granuloma formation in the lung [88]. However, antibody administration did not prevent the establishment of infection or dissemination from the lung. Although protection was partial, these studies provide strong encouragement for the continued development of conjugate vaccines which protect against *C. neoformans* infection by eliciting strong antibody immunity.

The major uncertainty regarding the conjugate vaccine approach is whether immunization of HIV-infected patients will elicit antibody responses that are quantitatively and qualitatively adequate to prevent *C. neoformans* infection. The experience with the *H. influenzae* conjugate suggests that antibody response to immunization will be dependent on the immunological status of the host [89]. One approach may be to immunize patients early in the course of HIV infection while their immune responses are relatively intact and hope that resulting antibody immunity persists if the

patient subsequently becomes immunosuppressed. The level of antibody that would be necessary to prevent infection in a patient with defective cellular immunity is not known. Clearly, resolution of issues of administration, efficacy, dose, adjutants, etc., await clinical trials.

The experience with the GXM-TT conjugate vaccine indicates that: (i) covalent linkage of cryptococcal polysaccharide to a carrier protein results in a highly immunogenic compound which can elicit high titre antibody responses to polysaccharide epitopes [76,77], (ii) protective and non-protective antibodies can be elicited by immunization [85,90], and (iii) the antibody response in mice [80,85], and possibly in humans [83], is restricted in terms of variable region utilization and idiotype.

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